REMARKS

1. **Status of the Application**

Claims 7-24 are pending in the present application. The following rejections are at issue and are set forth by number in the order in which they are addressed:

1) Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Cain et al. (WO 97/18320); and

2) Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Pariza et al. (U.S. Pat. No. 5,856,149).

A *prima facie* case of obviousness requires the Examiner to cite a combination of references which (a) disclose the elements of the claimed invention, (b) suggests or motivates one of skill in the art to combine those elements to yield the claimed combination, and (c) provides a reasonable expectation of success should the claimed combination be carried out. Failure to establish any one of the these three requirements precludes a finding of a *prima facie* case of obviousness, and, without more, entitles Applicant to allowance of the claims in issue.[1] In addressing this rejection, Applicants focus on the independent claims since non-obviousness of an independent claim necessarily leads to non-obviousness of claims dependent therefrom.[2] Applicants respectfully note that neither of the references teaches each element of the invention as claimed.

1. Cain et al. Does Not Teach Each Element of the Claims

Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Cain et al. (WO 97/18320). The Examiner repeats his argument that Cain et al. teaches a conjugated linoleic acid composition "of which 49.7% was the cis 9, trans 11-isomer and 50.3% was the trans 10, cis 12-isomer." (Office Action, page 2). In response to the Examiner's, arguments, applicants respectfully submit the Declaration of Mr. Asgeir Sæbo.

Mr. Sæbo repeated the conjugation conditions described in Cain et al. The results of the conjugation reactions were analyzed by GC-MS and are attached at Tab 1 to the Declaration. The conjugation method of Cain et al. resulted in a conjugated linoleic acid composition comprising approximately 3.49% c11,t13 CLA and 2.24% t9,t11and t10,t12 CLA. As Mr. Sæbo notes, the t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to

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1 *See, e.g., Northern Telecom Inc. v. Datapoint Corp.*, 15 USPQ2d 1321, 1323 (Fed. Cir. 1990).

2 §MPEP 2143.03.

one proportion to the c11,t13 isomer.

This factual evidence establishes that the methods utilized by Cain et al. do produce a variety of isomers other than the desired t10,c12 and c9,t11 isomers. Furthermore, Mr. Sæbo explains why the formation of the 8,10 and 11,13 isomers is a **necessary** result of the process employed by Cain et al. In particular, the formation of these isomers occurs by a process known as thermal sigmatropic rearrangement. This process is described in book chapter authored by Mr. Sæbo and attached as Tab 3 to the Declaration. As Mr. Sæbo states:

> [T]he research described in this chapter establishes that the formation of the 8,10 and 11,13 isomers is a necessary consequence of heating compositions containing the t10,c12 and c9,t11 isomers. Thus, whenever compositions containing t10,c12 and c9,t11 CLA are heated at temperatures such as those used by Cain et al. (i.e., 180°C for about 2 - 2.5 hours), 8,10 and 11,13 isomers are necessarily produced. Because Cain et al. does not describe the presence of these isomers in their compositions, the only reasonable conclusion is that they did not analyze for these isomers or chose to delete these isomers from their report because they were not considered to be active isomers.

Thus, the compositions of Cain et al. necessarily contained 8,10, and 11,13 isomers. As Mr. Sæbo goes on to state, the failure of Cain et al. to report these isomers "is explainable by the facts that 1) methods for the analysis of CLA compositions in 1996 were rather crude and 2) Cain may have simply chosen not to include non-active isomers when reporting their results."

Based on the foregoing arguments supported by factual evidence, Applicants respectfully submit that the Examiner has not established a prima facie case of obviousness (and that, assuming arguendo, a prima facie case of had been established, it now stands rebutted).

2. Pariza et al. Does Not Teach Each Element of the Claims

Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Pariza et al. (U.S. Pat. No. 5,856,149). The claims as currently pending require that the composition contain 10,12-octadecadienoic acid. Pariza et al. specifically teach at column 4, lines 36-39 that their composition does not contain the t10,c12 isomer. This interpretation of Pariza is confirmed by Mr. Sæbo in paraghraph 6 of his declaration. Thus, Pariza does not teach each element of the claims.

The Examiner refers to column 2, lines 50-58 of Pariza to support his arguments. It is respectfully noted that Pariza only addresses c9,t11 and t10,c12 isomers in this cited passage. The passage is silent with respect to other isomers. The statement that the compositions contain

about 42% each of the two isomers does not exclude the presence of other isomers. As established in Mr. Sæbo's Declaration, these isomers are **necessarily present in chemically processed plant oils.** Pariza simply does not teach each element of the claims and the claims should be passed to allowance.

In view of Pariza's silence with respect to the 8,10 and 11,13 isomers, it is apparent that the Examiner is relying on conclusory reasoning to modify the reference. The Examiner's resoning is conclusory because the reference is merely recited and then the Examiner states that the teaching of a compositions with about 42% of the c9,t11 and t10,c12 isomers makes the invention obvious. The Examiner fails to provide any reasoning as to why the 8,10 and 11,13 isoemrs would not be expected to be found in the remaining 16% of the composition. The Federal Circuit has expressly forbidden this approach hindsight-based approach.

Specifically, the Federal Circuit held that:

> The factual inquiry whether to combine references must be thorough and searching. It must be based on **objective evidence** of record. **This precedent has been reinforced in myriad decisions, and cannot be dispensed with.**

See, In re Lee, 277 F.3d 1338, 1344 (Fed. Cir. 2002); internal citations omitted; emphasis added. Indeed, the Federal Circuit has made it clear that "[b]road, **conclusory** statements regarding the teachings of multiple references, standing alone, are not 'evidence.'" *In re Dembiczak*, 175 F.3d 994, 50 USPQ2d 1614 (Fed. Cir. 1999)(emphasis added).

Thus, the Examiner's conclusory motivation statement falls well short of the standards established by the Federal Circuit. In particular, the Examiner has provided no rationale as to why someone skilled in the art would not expect the remaining 16% of the composition to contain the 8,10 and 11,13 isomers. Given this fact, it is apparent that the Examiner has applied hindsight reconstruction to reject the claims. This is the situation that the above standards are meant to prevent:

> The Board did not . . . explain what specific understanding or technological principal within the knowledge of one of ordinary skill in the art would have suggested the combination. **Instead, the Board merely invoked the high level of skill in the art.** If such a rote invocation could suffice to supply a motivation to combine, the more sophisticated scientific fields would rarely, if ever, experience a patentable technological advance. Instead, in complex scientific fields, the Board could routinely identify the prior art elements in an application, invoke the lofty level of skill, and rest its case for rejection. To counter this potential weakness in the obviousness construct, the suggestion to combine requirement stands as a critical safeguard against hindsight analysis and rote

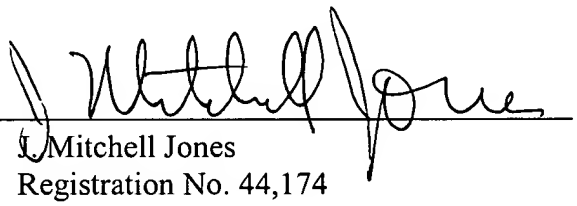
application of the legal test for obviousness (Emphasis added).

In re Rouffet, 47 USPQ2d 1453 (Fed. Cir. 1998). Accordingly, Applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of obviousness because the Federal Circuit standards for motivation to modify have not been met. As such, the obviousness rejection should be removed.

Conclusion

All grounds of rejection and objection of the Office Action of August 28, 2003 having been addressed, reconsideration of the application is respectfully requested. It is respectfully submitted that the invention as claimed fully meets all requirements and that the claims are worthy of allowance. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicant encourages the Examiner to call the undersigned collect at (608) 218-6900.

Dated: November 25, 2003



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Asgeir Sæbo *et al.*		
Serial No.:	09/438,104	Group No.:	1614
Filed:	November 10, 1999	Examiner:	Jones, D.
Entitled:	**Conjugated Linoleic Acid Compositions**		

Declaration of Asgeir Sæbo

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: 11-25-03 By: Susan M. McClintock
Susan M. McClintock

I, Asgeir Sæbo, state as follows:

1. My present position is Vice President of Technology, Natural ASA.

2. I have reviewed the above captioned patent application, of which I am an inventor, the Office Action mailed April 10, 2002, and the Cain and Pariza references cited as prior art.

3. In the repeat of Cain, the conjugation conditions were the same as those described in Example 6 of WO97/18320. The results of the conjugation reactions were analyzed by GC-MS. The results are attached at Tab 1. As can be seen, this conjugation method resulted in a conjugated linoleic acid composition comprising approximately 3.49% c11,t13 CLA and 2.24% t9,t11and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer. I note that this method is very similar to the method utilized in the Sugano reference, which was discussed in my previous Declaration. My work confirms that these methods produce CLA with relatively high levels of undesirable isomers.

4. The Examiner states at page 4 of the Office Action that Cain teaches CLA compositions

that are composed of 49.7% c9,t11 and 50.3% t10,c12 CLA, and that because these numbers add up to 100% no other isomers were present. However, the percentages reported do not mean that the other isomers were not present, as was found in my repeat of Cain. This discrepancy is explainable by the facts that 1) methods for the analysis of CLA compositions in 1996 were rather crude and 2) Cain may have simply chosen not to include non-active isomers when reporting their results. Improved methods for detecting the various isomers of CLA were not developed until well after the 1995 priority date of Cain. This fact is substantiated by Yurawecz *et al.* (attached at Tab 2), who state "the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies [including gas chromatography techniques] that have only recently been developed." (Yurawecz, *p.* 281). Thus, Cain et al. may not have conducted an analysis that could detect the isomers in questions. Consideration of Example 18 of Cain et al. supports this analysis. The inventors state that their compositions, produced by the method of Example 6, contained 63.8% CLA, of which 48.9% was the cis 9, trans 10 isomer and 51.1% was the trans 10, cis 12 isomer. This means that the inventors provide no analysis of the remaining 36.2% of their composition. The 8,10; 11,13; and trans-trans isomers that are discriminated against in the present invention and detected in my repeat of Cain could well have been present in this fraction.

5. I further note that the formation of the c11,t13 isomer from the t10,c12 isomer and the t8,c10 isomer from the c9,t11 isomer is caused by a process known as thermal sigmatropic rearrangement. This process is described in Chapter 5 of the book Advances in *Conjugated Linoleic Acid Research, Volume 2*, J. Sebedio, W.W. Christie, and R. Adolf, Eds., AOCS Press, Champaign, IL, 2002. I wrote this chapter. Briefly, this research described in this chapter establishes that the formation of the 8,10 and 11,13 isomers is a necessary consequence of heating compositions containing the t10,c12 and c9,t11 isomers. Thus, whenever compositions containing t10,c12 and c9,t11 CLA are heated at temperatures such as those used by Cain et al. (i.e., 180°C for about 2 - 2.5 hours), 8,10 and 11,13 isomers are necessarily produced. Because Cain et al. does not describe the presence of these isomers in their compositions, the only reasonable conclusion is that they did not analyze for these isomers or chose to delete these isomers from their report because they were not considered to be active isomers.

6. With regard to the rejection over Pariza (U.S. Pat. No. 5,856,149), I note that Pariza et al. does not teach compositions that contain both c9,t11 and t10,c12 isomers while containing less than 1% 8,10 and 11,13 isomers.

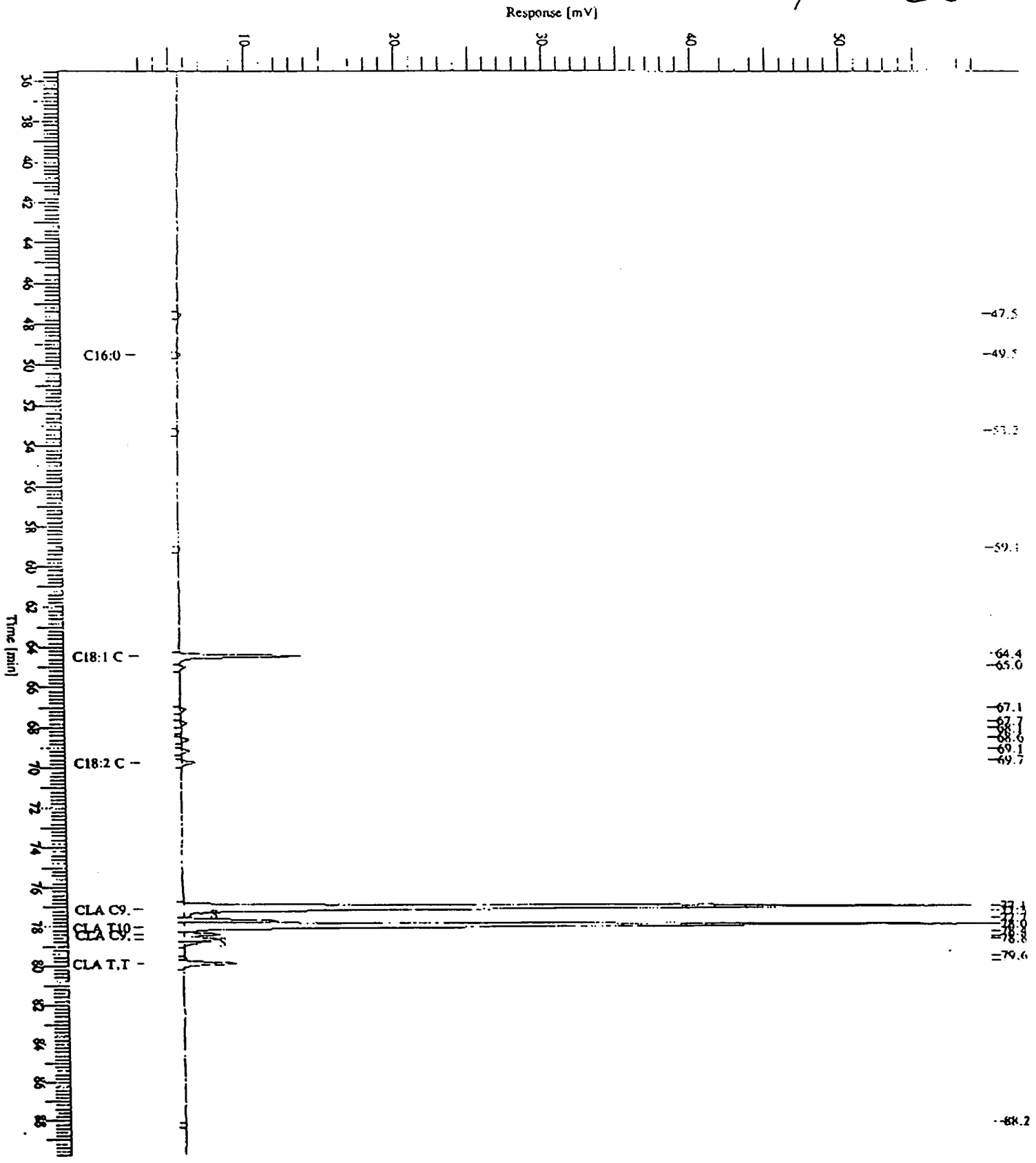
7. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Asgeir Sæbo

Date: Oct 31 2003

Sample Name : 6659: A01348, 024/96-1, CLA FFA Sample #: 001 Page 1 of 1
FileName : D:\TCWS Data\data\Data 100E 1000-19...\100e1001.raw
Date : 19.11.01 11:48:38
Method : Time of Injection: 19.11.01 09:08:32
Start Time : 35.53 min End Time : 89.83 min Low Point : 2.75 mV High Point : 59.51 mV
Plot Offset: 2.75 mV Plot Scale: 56.8 mV

WO 97/18320



Software Version : 6.1.2.0.1:D19
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Instrument Name : GC
Rack/Vial : 0/1
Sample Amount : 1.000000
Cycle : 1
Date : 19.11.01 11:48:37
Data Acquisition Time : 19.11.01 09:08:32
Channel : B
Operator : Operatør
Dilution Factor : 1.000000

Result File : D:\TCWS Data\data\Data 100E 1000-1999\100e1001.rst
Sequence File : D:\TCWS Data\sekvenser\100E.20.10.00..seq

FATTY ACID PROFILE REPORT

PERKIN ELMER AUTOSYSTEM XL GC

Column: WCOT FUSED SILICA 100 m x 0.25 mm COATING CP-SIL 88 DF= 0.2 Chrompack cat.no: 7489
Carrier Gas: He, 30.0 PSI
Method: 100E.mth
Temp: 80 C (2 min)->45 C/ min->130 C (0 min)->1 C/ min-> 220 C (10 min)
Injection: Splitless, 240 C
Detector: FID, 280 C

Peak #	Time [min]	Component Name	Area [%]	Area [µV·s]	Height [µV]
1	47.557		0.14	2040.57	221.66
2	49.507	C16:0	0.12	1770.08	234.26
3	53.277		0.07	1043.10	118.41
4	59.139		0.07	1079.52	131.55
5	64.461	C18:1 c9	4.84	72109.91	8053.81
6	65.035		0.23	3435.33	396.61
7	67.125		0.25	3718.15	401.86
8	67.795		0.28	4195.57	459.60
10	68.621		0.31	4688.64	520.82
11	69.176		0.33	4880.16	532.98
12	69.744	C18:2 c9,c12	0.53	7977.36	868.60
13	77.128	CLA c9,t11+t8,c10	42.84	638739.60	52812.75
14	77.371		0.28	4120.52	216.07
15	77.752	CLA c11,t13	3.49	51987.22	6233.41
16	78.067	CLA t10,c12	40.35	601682.23	54289.00
17	78.437	CLA c9,c11	1.36	20327.77	2373.19
18	78.664	CLA c10,c12	1.61	24007.50	2280.68
19	78.808		0.58	8661.37	1107.38
20	79.693		0.08	1265.48	173.63
21	79.909	CLA t,t 9,11+10,12	2.24	33420.59	3512.11
			100.00	1491150.67	134938.38

Missing Component Report

Component	Expected Retention (Calibration File)
C18:0	0.001

19.11.01 11:48:37 Result: D:\T\ /S Data\data\Data 100E 1000-1999\100e1001.rst

Analyzed by: Natural ASA, Hovdebygda

Approved by: _____

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Lipid Journal for Science and Technology of Fats, Oils and Waxes

Inhalt

Contents

Variations in isomer distribution in commercially available conjugated linoleic acid*

Martin P. Yurawecz[1], Najibullah Sehat[1], Magdi M. Mossoba[1], John A.G. Roach[1], John K. G. Kramer[2], and Youh Ku[1]

Conjugated linoleic acid (CLA) has been reported to have anticarcinogenic and antiatherogenic properties, to repartition body fat, to build bone mass, to normalize glucose tolerance, and to reduce hyperglycemia and diabetes. CLA products are now commercially available, and there is considerable interest in studying CLA because of this range of reported beneficial effects. However, little is known about the composition of these preparations. Representative commercial CLA products in capsule or liquid (aqueous or oily) form were analyzed for their CLA content and isomer composition using gas chromatography (GC), silver ion-high performance liquid chromatography (Ag^+-HPLC) and spectroscopic techniques. The content of CLA in the preparations varied widely. Based on the GC-internal standard technique, total CLA varied from 20 to 89% by total weight and 28 to 94% of total fat. One product contained no CLA. The isomer distributions were generally of two types: those with two major CLA positional isomers, and those with four major CLA positional isomers. All the CLA preparations in capsule form contained the four isomer mixture, while the liquid preparations contained from two to four CLA positional isomers.

Unterschiede in der Isomerenverteilung kommerziell erhältlicher konjugierter Linolsäure. Von konjugierter Linolsäure (CLA) wurde berichtet, daß sie anticanzerogene und antiatherogene Eigenschaften hat, Körperfett repartioniert, Knochenmasse aufbaut, Glukosetoleranz normalisiert und Hyperglykämie und Diabetes reduziert. CLA-Produkte sind jetzt kommerziell erhältlich, und es gibt wegen der oben aufgeführten prositiven Effekte ein beträchtliches Interesse daran, CLA zu studieren. Allerdings ist wenig bekannt über die Zusammensetzung dieser Herstellungen. Repräsentative kommerzielle CLA-Produkte in Kapsel- oder flüssiger Form (auf Wasser- oder Ölbasis) wurden mit Hilfe eines Gaschromatographen (GC), Silberionen-Hochdruckflüssigkeitschromatographie (Ag^+-HPLC) und spektroskopischer Techniken auf ihren CLA-Inhalt und ihre isomere Zusammensetzung analysiert. Der Inhalt der CLA in den Herstellungen variierte stark. Auf der Basis der GC-internen Standardtechnik schwankten die gesamten CLA zwischen 20 und 89% bezogen auf das Gesamtgewicht und zwischen 28 und 94% bezogen auf den Gesamtfettanteil. Ein Produkt enthielt keine CLA. Die Isomerverteilungen untergliederten sich allgemein in zwei Typen: solche mit zwei Positionsisomeren und solche mit vier Positionsisomeren. Alle CLA-Herstellungen in Kapselform beinhalteten das Gemisch der vier Isomere, während die flüssigen Herstellungen zwischen zwei und vier der Positionsisomere enthielten.

1 Introduction

Conjugated linoleic acid (CLA) has been reported to provide direct [1] or indirect [2] protection against several types of cancer, atherosclerosis [3, 4], and diabetes [5]. CLA has also been reported to improve feed efficiency [6] and increase muscle [7, 8], and bone mass [9]. These results were generally obtained in experimental animals fed commercial CLA preparations containing approximately equal amounts of four *cis/trans* conjugated octadecadienoic (18:2) acids: 8 *trans*, 10 *cis*-18:2; 9 *cis*, 11 *trans*-18:2; 10 *trans*, 12 *cis*-18:2; 11 *cis*, 13 *trans*-18:2; and minor amounts of the corresponding *cis,cis* and *trans,trans* CLA isomers [10]. Thus, the contribution(s) of the specific isomers to the observed effects are not known. In contrast, natural products, such as milk, cheese, and meat from ruminant animals contain mainly rumenic acid (9 *cis*, 11 *trans*-18:2) [11–13] with minor amounts of 7 *trans*, 9 *cis*-18:2 [14] and other isomers [15–17]. The total CLA content in these natural products ranges from trace to 2% of total fatty acids [12, 18, 19].

The present study was undertaken to determine the content and distribution of CLA isomers in commercially available CLA capsules and liquid products with labels stating to contain CLA. The CLA isomers were analyzed by gas chromatography (GC) and silver ion-high performance liquid chromatography (Ag^+-HPLC) as their fatty acid methyl esters (FAME), and identified by GC-electron ionization mass spectroscopy (GC-EIMS) and GC-direct deposition-Fourier transform infrared (GC-DD-FTIR) spectroscopy as their 4,4-dimethyloxazoline derivatives [10, 14, 17, 20].

2 Materials and Methods

2.1 Chemicals

Representative CLA preparations were purchased locally, from specialty chemical companies, or from the World Wide Web. Several pure CLA isomers were obtained as their free fatty acids from *Matreya*, Inc. (Pleasant Gap, PA). Acetonitrile and hexane were UV grade. Other solvents were distilled-in-glass quality. 2-Amino-2-methyl-1-propanol (95%) was purchased from *Aldrich Chemical Company*, Inc. (Milwaukee, WI). A 10% solution of trimethylsilyldiazomethane in hexane was obtained from *TCI America* (Portland, OR).

[1] US Food and Drug Administration, Center for Food Safety and Applied Nutrition, USA.
[2] Southern Crop Protection, Food Research Center, Agriculture and Agri-Food Canada, Canada.
* Presented in part at the 52nd International Deutsche Gesellschaft für Fettwissenschaft (DGF) Congress in Magdeburg, Germany, Sept. 13–15, 1998.

Anhydrous $NaOCH_3$/methanol was purchased from *Supelco*, Inc. (Bellefonte, PA.).

2.2 Lipid extraction

A known weight (approximately 25 mg) of each product was dissolved in 2 ml 1 N KOH in ethanol (95%) and hydrolyzed overnight in the dark at room temperature. For quantitative analyses, one mg of eicosanoic acid (23:0) was added as an internal standard. After hydrolysis, 5 ml of H_2O and one ml of 6 N HCl were added and the free fatty acids were extracted three times with 5 ml diethyl ether/petroleum ether (1:1). The combined extracts were washed with H_2O and dried over anhydrous Na_2SO_4, and the solvents removed under a stream of argon. Aqueous CLA samples were first extracted with petroleum ether/diethyl ether (1:1), and 25 mg of the extracted lipids were treated as described above.

2.3 Derivatizations

FAMEs were prepared for GC by dissolving the free fatty acids in one ml of benzene/methanol (4:1) to which 0.5 ml of a 10% solution of trimethylsilyldiazomethane in hexane were added [21]. The reaction was allowed to stand for 0.5 h with occasional gentle shaking. Thereafter, five drops of glacial acetic acid were added with gentle shaking. The same amount of glacial acetic acid was added to each of the solutions to destroy excess yellow trimethylsilyldiazomethane. Some solutions did not become clear on addition of glacial acetic acid. Then 5 ml of H_2O were added, and the reaction mixture was extracted with one ml of isooctane. The extract was subsequently dried over anhydrous Na_2SO_4.

The 4,4-dimethyloxazoline (DMOX) derivatives were prepared to determine the double bond position of CLA isomers. Ten to 20 mg of the free fatty acid product prepared above was added to a screw cap reaction tube (1 ml) and a threefold excess (w/w) of 2-amino-2-methyl-1-propanol was added. The tube was purged with argon, capped, and heated at 170 °C for 0.5 h in an oven. DMOX derivatives were then partitioned into petroleum ether as described previously [22].

2.4 Gas chromatography

The analyses of the FAMEs were carried out using a *Hewlett-Packard* (Palo Alto, CA) model 5890 gas chromatograph fitted with a flame-ionization detector. A CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; *Chrompack*, Bridgewater, NJ) was used, and H_2 was the carrier gas at a split ratio of 50:1. The column was operated at 75 °C for 2 min, then temperature-programmed at 5 °C/min to 185 °C, held for 30 min, followed by a second temperature program at 4 °C/min to 225 °C and held there for 33 min.

2.5 Ag^+-HPLC

The HPLC (*Waters 510* solvent delivery system; *Waters Associates*, Milford, MA) was equipped with an autosampler and 200-µl injection loops (*Waters 717*), a UV detector operated at 233 nm (*Waters 486* tunable absorbance), and a data system (*Waters Millennium*™ version 2.15). A *ChromSpher 5 Lipids* analytical silver impregnated column (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size; *Chrompack*, Bridgewater, NJ) was operated at room temperature. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 ml/min. The retention times varied slightly between runs due to the insolubility of acetonitrile in hexane. However, these changes did not affect the relative elution sequence of CLA isomers. Typical injection volumes were 5–15 µl at a concentration of 1 mg total FAME per ml.

2.6 Gas chromatography – electron ionization mass spectrometry

The GC-EIMS analyses were performed by using a *Hewlett-Packard* (model 5890, series II) GC coupled to a mass spectrometer (*Autospec Q* mass spectrometer) and a data system (*OPUS 4000; Micromass*, Manchester, UK). The GC-EIMS system utilized version 2.1 BX software. This system was used with a 50 or 100 m CP-Sil 88 fused-silica capillary column. The GC-EIMS conditions were: splitless injection with helium or hydrogen as the carrier gas and sweep was restored 1 min after injection. The injector and transfer lines temperatures were 220 °C. The column was operated at 75 °C for one min after injection, then temperature-programmed 20 °C/min to 185 °C, held there for 15 min, then temperature-programmed 4 °C/min to 220 °C, and held there for 45 min.

Tab. 1. Conjugated linoleic acid (CLA) methyl ester isomers, as % of total CLA, in 13 commercial CLA preparations as determined by silver ion-high performance liquid chromatography (Ag^+-HPLC).

Product	*trans,trans*				*cis/trans*[a]				*cis,cis*			
	11,13	10,12	9,11	8,10	11,13	10,12	9,11	8,10	11,13	10,12	9,11	8,10
1 aqueous	0[b]	0	0	0	0	0	0	0	0	0	0	0
2 oil	0	1.1	1.0	tr[c]	0	47.1	50.8	tr	0	tr	tr	0
3 oil	0	0.5	0.5	0	1.1	50.2	47.6	tr	0	tr	tr	0
4 oil	0	1.1	1.3	0	0	45.8	50.7	tr	0	1.1	0.1	tr
5 oil	0	0.6	0.6	0	0	54.0	43.5	0	0	0.7	0.6	0
6 oil	1.5	5.4	11.9	7.1	2.2	38.3	21.7	tr	0	2.3	6.2	3.5
7 capsule	0.7	2.7	2.8	0.5	19.0	32.1	25.6	15.6	0.7	0.4	tr	tr
8 capsule	0.8	2.7	2.5	0.4	16.8	33.9	27.1	14.2	tr	1.7	tr	tr
9 capsule	1.0	3.1	2.8	0.5	16.9	33.7	26.9	14.0	0.6	0.5	tr	tr
10 capsule	0.7	2.5	2.5	0.6	15.5	31.0	27.7	14.2	0.8	2.2	1.8	0.6
11 capsule	0.4	2.8	2.9	0.5	14.4	29.1	30.0	15.9	0.5	1.5	2.1	tr
12 capsule	1.3	3.3	3.4	1.1	19.8	25.9	21.6	16.5	1.2	2.7	2.4	1.0
13 oil	4.0	5.4	5.4	1.7	19.7	26.8	25.6	10.5	0.2	0.1	0.6	0.1

[a] The CLA isomers exist either in the *cis,trans* or *trans,cis* configuration. [b] 0, not detectable. [c] tr, trace (<0.05%).

2.7 GC-direct deposition Fourier transform infrared spectroscopy

GC-DD-FTIR was performed using a *Bio-Rad* (Cambridge, MA) *Tracer™ GC-FTIR 60A* spectrometer system. This system was used with a 50 m CP-Sil-88 fused-silica capillary column as described previously [23, 24].

3 Results and Discussion

Preparations of CLA were capsules or liquids that were water-based or oil-based; some contained non-lipid material. Their chemical compositions were not known. Based on the assumption that the CLA products consisted of esters, free fatty acids or combinations thereof, all products were hydrolyzed under alkali conditions and subsequently methylated by using trimethylsilyldiazomethane as catalyst to ensure preservation of the original distribution of CLA isomers [25].

Total fatty acid compositions of the 13 CLA products were determined. The internal standard (23:0) added to the CLA products provided a mean to determine the amount of total fatty acids in the original CLA mixture. When this total fatty acid value was compared to the 25 mg of starting material used, an approximate estimate of the non-lipids in the sample was obtained. The approximate amount of non-lipid material calculated for these products ranged from 3 to 28%. In addition, unidentified FAMEs ranged from 1 to 29%. The

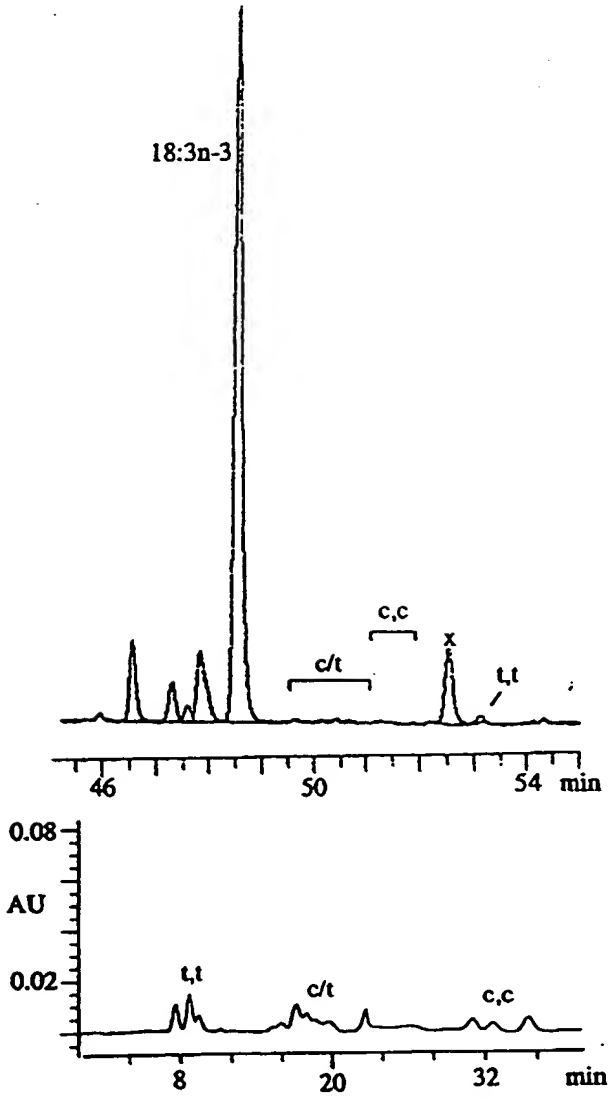

Fig. 1. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a commerial conjugated linoleic acid (CLA) preparation containing no CLA. The corresponding CLA regions in each chromatogram are labelled; x is an unknown component. The absorbance scale is shown in the lower graph to indicate the low response found in the CLA region.

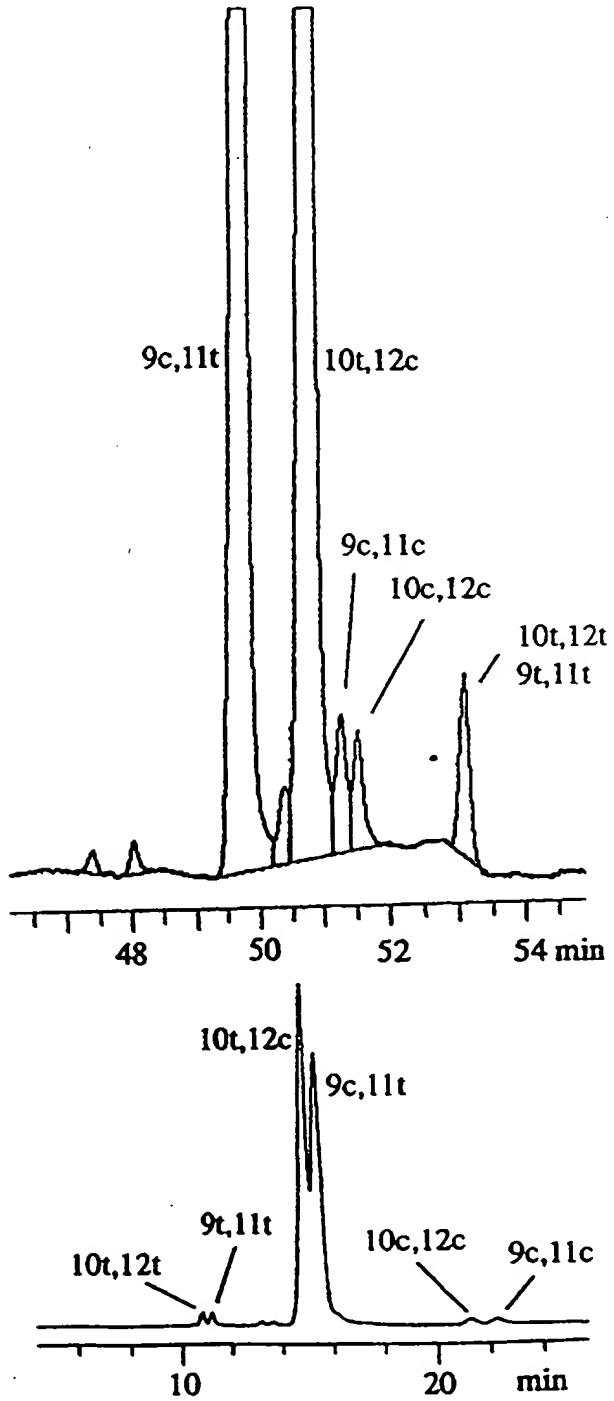

Fig. 2. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a representative commerial conjugated linoleic acid (CLA) preparation consisting primarily of two CLA isomers, 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2. Peaks corresponding to CLA isomers in each chromatogram are labelled.

major fatty acids, other than the CLA isomers found in the preparations, included palmitic (16:0), stearic (18:0), oleic (18:1n-9), and linoleic (18:2n-6) acids. Their combined content ranged from 1 to 84% in the products examined. Based on the GC-internal standard technique, total CLA content ranged from 0 to 94% of the total FAMEs, or 0 to 89% of the mass content of the products.

The CLA isomer distributions in the products, analyzed by a combination of GC and Ag^+-HPLC, are shown in Tab. 1. No CLA was found in product 1. The CLA-containing products fell into two catagories: those composed of two major CLA positional isomers (9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2), and those composed of four major CLA positional isomers (8 *trans*, 10 *cis*-18:2; 9 *cis*, 11 *trans*-18:2; 10 *trans*, 12 *cis*-18:2, and 11 *cis*, 13 *trans*-18:2). Other minor CLA isomers were present at much lower concentrations, but are not reported. All the CLA products in capsule form contained the mixture of four isomers, while the liquid products contained either two or four CLA positional isomers. Representative GC and Ag^+-HPLC chromatograms for these groups are shown in Figs. 1, 2, and 3, respectively.

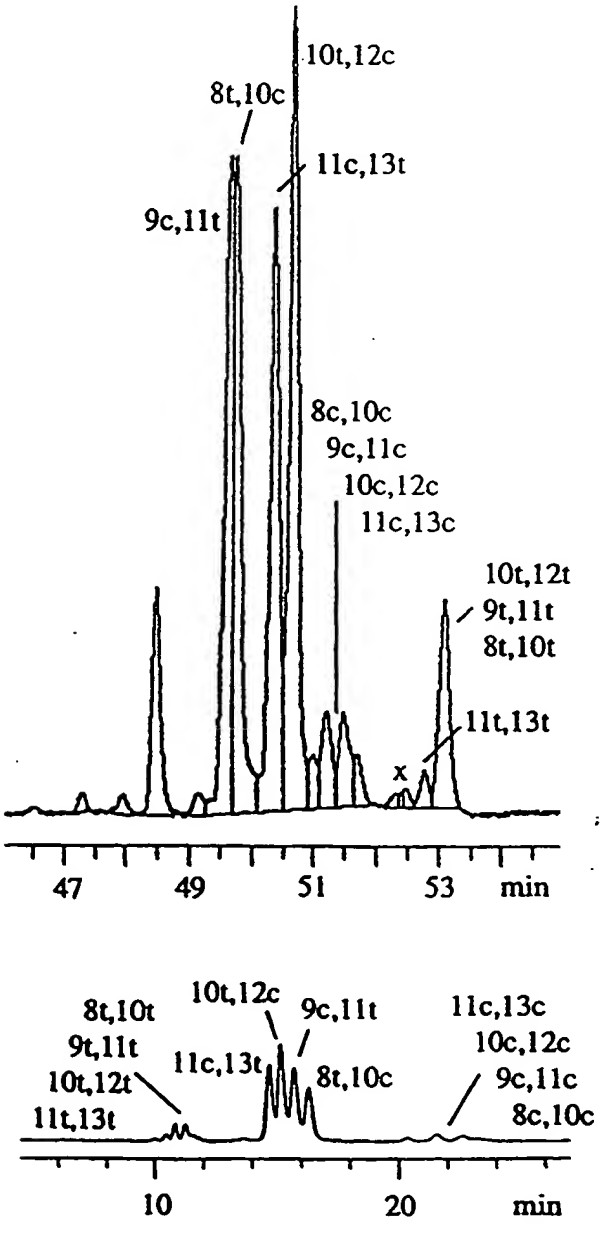

Fig. 3. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a representative commerial conjugated linoleic acid (CLA) preparation consisting primarily of four CLA isomers, 8 *trans*, 10 *cis*-18:2, 9 *cis*, 11 *trans*-18:2, 10 *trans*, 12 *cis*-18:2 and 11 *cis*, 13 *trans*-18:2. Peaks corresponding to CLA isomers in each chromatogram are labelled; x is an unknown component.

An explanation for the differences in isomer distributions among the products was not available. Alkali isomerization of 18:2 n-6 in laboratory scale batches prepared according to published procedures [12, 26, 27] resulted in the formation of only two CLA isomers, i.e., 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2. Alkali isomerization under large-scale, and possibly more severe conditions, may have produced the four CLA positional isomer pattern observed in the commercial CLA preparations described here. This will need to be confirmed.

The GC analyses were based on use of a 100 m polar capillary column. In this system, 8 *trans*, 10 *cis*-18:2 was not resolved from 9 *cis*, 11 *trans*-18:2. A shoulder or a split peak may occasionally be evident when the amounts of these two isomers are approximately equal. In contrast, 11 *cis*, 13 *trans*-18:2 eluted before and was resolved from 10 *trans*, 12 *cis*-18:2 using this GC column (Fig. 3, upper graph). The *cis,cis* CLA isomers eluted after 10 *trans*, 12 *cis*-18:2 in the order 8, 10-, 9, 11-, 10, 12-, and 11,13-18:2 as established previously [10, 20]. The last CLA isomers to elute were the *trans,trans*, consisting of a small peak due to 11,13-18:2 followed by an unresolved mixture of 10,12-, 9,11-, and 8,10-18:2 as demonstrated previously [14, 17]. A small unknown peak was observed between the *cis,cis* and the *trans,trans* CLA isomer regions. The structural identity of all CLA isomers was established and confirmed by analyzing the DMOX derivatives of selective CLA products by GC-EIMS and GC-DD-FTIR. Representative mass and infrared spectra were published previously [10, 14, 17, 27].

Chromatograms showing the separation of the CLA isomers by Ag^+-HPLC are presented below the GC chromatograms in Figs. 1 to 3. The elution orders of all the geometric (in the order *trans,trans*, *cis/trans*, and *cis,cis*) and positional (in the order 11,13-, 10,12-, 9,11-, and 8,10-18:2) CLA isomers by Ag^+-HPLC were established previously [10]. Ag^+-HPLC was essential to complement the GC analysis and establish the composition of 8 *trans*, 10 *cis*-18:2 and 9 *cis*, 11 *trans*-18:2, and the distribution of most of the *trans,trans* CLA isomers.

In contrast to the commercial CLA preparations, that were found to contain two or four CLA positional isomers, natural dairy products and meats from ruminant animals contain primarily rumenic acid, 9 *cis*, 11 *trans*-18:2 [11, 12, 14, 17–19]. While it has not been established, which isomer(s) is (are) responsible for the reported beneficial properties of CLA, it is generally thought that anticarcinogenicity is due to rumenic acid [12, 15]. The nutritional and physiological effects, if any, of other CLA isomer(s) in commercially available CLA preparations are not known.

We recently reported, that one of the four major *cis/trans* CLA isomers, 11 *cis*, 13 *trans*-18:2, accumulates preferentially in heart phospholipids and specifically in heart and liver diphosphatidylglycerol (DPG) f pigs fed a CLA mixture containing four positional isomers [20]. DPG is a major component of inner mitochondrial membranes and is involved in many enzymes of bioenergetics in the mitochondria [28, 29]. *Watkins* et al. [30] demonstrated that docosahexaenoic acid (22:6 n-3) accumulated in DPG of human colonic adrenocarcinoma (HT-29) cells and increased mito-

chondrial oxidant production. Similarly, 11 *cis*, 13 *trans*-18:2 (or any other CLA isomer incorporated into DPG), could affect mitochondrial oxidant production, particularly since it has been shown that the oxidative susceptibility of CLA is comparable to that of arachidonic acid (20:4 n-6) [31, 32]. In response to our findings that 11 *cis*, 13 *trans*-18:2 was selectively incorporated into DPG [20], a major supplier of commercial CLA preparations recently modified the process to eliminate the 11 *cis*, 13 *trans*-18:2 isomer. The simultaneous elimination of 8 *trans*, 10 *cis*-18:2 from the resulting CLA mixture was an additional benefit of preparing a CLA mixture devoid of 11 *cis*, 13 *trans*-18:2.

In conclusion, the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies that have only recently been developed [10, 14, 16, 17, 20, 27]. All commercially available CLA products investigated differ, some significantly, and the isomers present may not necessarily represent active CLA components. As new products consisting of two or perhaps only one CLA isomer become available, it will be possible to determine the physiological effects of specific isomers. This is essential for an understanding of this unusual group of lipids.

Abbreviations

Ag^+-HPLC, high-performance liquid chromatography; *cis/trans*, refers to all the CLA isomers having either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC-DD-FTIR, gas chromatography-direct deposition-Fourier transform infrared; GC-EIMS, gas chromatography-electron ionization mass spectrometry.

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Advances in Conjugated Linoleic Acid Research, Volume 2

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Chapter 5

Commercial Synthesis of Conjugated Linoleate

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Introduction

Conjugated linoleic acid (CLA) has been available as a health food supplement in soft gelatine capsules since 1995 in the United States, and more recently in several European countries and Japan. CLA products designed for food and animal feed additives are expected to appear on the market in the near future. CLA has been produced for decades for technical purposes and continues to be used as a substitute for Chinese tung oil in the paint and varnish industry due to its "drying" characteristics. The production methods developed for technical CLA products were rapidly modified and improved upon after the discovery of the biological activity of the substance. This chapter will focus on supplements in particular, including current production methods, stability, and breakdown products. Purified isomers are currently available only for research purposes, but a few references to methods available for purification will be given.

CLA for Technical Applications

Dehydration of Ricinoleic Acid

Several decades ago, only two natural oils (tung oil and oiticica) were known to contain conjugated double bonds. Oils that contain these bonds rapidly form a polymer film ("drying") if a thin layer is exposed to air; tung oil was widely used in the paint and varnish industry. An increasing demand for such oils and limited availability encouraged efforts to produce drying oils from nonconjugated oils.

The main constituent of castor bean oil is ricinoleic acid (12-hydroxy-9-octadecenoic acid). Around 1937, dehydrated castor oil appeared on the market in the United States as a substitute for tung oil. Ten years later the product was established as one of the most popular drying oils (1). It has been known since 1888 that castor oil could be dehydrated, and since 1914 it was known that the main isomers of linoleic acid formed had double bonds at positions 9,11 and 9,12, but the detailed composition of dehydrated ricinoleic acid was not investigated until recently. A German patent from 1930 (2) and a U.S. patent from 1934 (3) describe the preparation of dehydrated castor bean oils. A modified procedure was recently used to produce an 83% pure 9-*cis*,11-*trans* CLA concentrate from purified ricinoleic acid (4). Main impurities were the 9-*cis*,11-*cis* and 9-*cis*,12-*trans*-octadecadienoic acids. Conventional dehydration

using high temperatures will create other isomers, mainly 8-*trans*,10-*cis* and *trans*, *trans* isomers. CLA from dehydrated castor oil is not available on the market in supplement form. Apart from safety issues, the reason is the absence of 10-*trans*,12-*cis* CLA, the isomer shown to inhibit fat synthesis (5).

Alkali Isomerization of Linoleic Acid Oils

Attempts to produce drying oil from nonconjugated oils were successful in the late 1930s as well as for oils containing methylene-interrupted fatty acids. In 1941, a U.S. patent was issued that describes the use of monohydric and polyhydric alcohols as solvents and a variety of alkaline catalysts (6). A few years later, two patents were issued that described the use of water (7) and steam (8), respectively, as solvent in an autoclave to achieve the temperatures necessary to conjugate unsaturated acids. It is actually the soap that is conjugated. Upon addition of mineral acid, the conjugated free fatty acids are liberated. Currently, CLA is produced for technical purposes in high alkaline water at ~230°C. Feedstock is usually free fatty acids (after fat splitting to recover glycerol). The product is usually distilled to yield a virtually colorless oil.

Production of CLA for Animal and Human Consumption

Alkaline Water Isomerization

The first products to appear on the health food market contained ~65% CLA, and the profile of the CLA isomers was similar to technical-grade products. Christie *et al.* (9), showed that the main isomers of CLA in addition to 9-*cis*,11-*trans* and 10-*trans*,12-*cis* were an 8,10 and an 11,13 isomer *cis,trans* or *trans,cis*. These were later identified as 8-*trans*,10-*cis* and 11-*cis*,13-*trans* (10). Such products are still available as supplements, and most if not all are produced from linoleate-rich starting materials in high-alkaline water reactions at temperatures >230°C. We investigated reaction parameters in water alkaline (KOH or NaOH catalyst) reactions trying to avoid formation of 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. It turned out not to be possible to achieve a nearly quantitative isomerization and at the same time avoid formation of the said isomers (data not published).

Isomerization in Propylene Glycol

Quantitative isomerization of oils containing polyunsaturated fatty acids in monohydric and polyhydric alcohols was described in 1941 (6). A detailed procedure using ethylene glycol is described in a patent from 1996 (11). Ethylene glycol has not been used commercially for production of CLA for consumer safety reasons. Propylene glycol has therefore been selected by several producers who independently developed proprietary procedures (12,13). KOH was selected as catalyst because of its high solubility compared with NaOH. Reaction temperatures are typically 130–180°C, and times of reaction are from 3 to >24 h. The quantity of KOH

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Consumption

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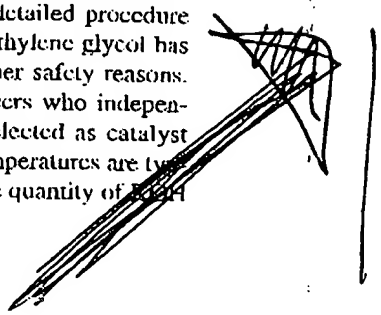

is substantial and in excess of that needed for quantitative saponification. After the reaction is complete, the mixture is cooled down and water and mineral acid (hydrochloric or sulfuric) are added. Free fatty acids of CLA are liberated as soon as the mixture becomes acidic. One patent describes the use of hexane at this point to extract CLA and facilitate separation from the bottom aqueous layer without emulsion problems. However, the operation is possible without the use of hexane. For the sake of recovery of propylene glycol, free fatty acids are preferred as feedstock oil. A triacylglycerol feedstock will create glycerol to contaminate the propylene glycol. After water and solvent (hexane if used) have been removed under vacuum, the CLA product is preferably purified by deodorization and distillation. Peroxides and volatiles are easily removed by deodorization. The peroxides are broken down to secondary volatile products that are removed in the process.

The purification process should also include a molecular distillation step to remove nonvolatile compounds such as polymers, sterols, and propylene glycol esters. Heavy metals could also arise from the isomerization process if mineral acids are used in stainless steel reactors (14). Their concentrations are reduced upon molecular distillation as well. A distilled product is almost colorless and has an acid value of ~200 (mg KOH/g). A nondistilled product might have an acid value of ~190, be yellow to slightly brown in color and have an opaque appearance. However, we have observed a slight decrease in acid value in capsules over time and also a darkening of the oil if the capsule material is colored. Due to the strong alkaline process, free fatty acids are the final product regardless of the form of feedstock (free fatty acid, a monoalkyl ester, or a triacylglycerol oil). Therefore, CLA in supplements are offered almost exclusively as free acids, in contrast to n-3 concentrates that are offered either as ethyl esters or reesterified triacylglycerols.

Isomerization of Mono-Alkyl Esters Using Alkali Metal Alcoholates

Recently, a proprietary method has been developed that quantitatively isomerizes methyl esters and ethyl esters of linoleic acid using very low quantities of catalysts and virtually no solvents (data not published). Because of the quantity of catalyst (~2%), only a small fraction of the ester is saponified and hence appears as free fatty acid after addition of a neutralizing agent. Most of the product (>92%) is still in the form of the methyl or ethyl ester after the isomerization process. The reaction proceeds at temperatures down to below 100°C, and the CLA product is characterized by very low levels of CLA isomers produced by thermal [1,5] sigmatropic rearrangements (see below).

Thermal [1,5] Sigmatropic Rearrangements of CLA Isomers

Production of CLA in propylene glycol or other alcohol under mild conditions gives rise to <0.5% each of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. After purification of single isomers, we showed that upon heating to 220°C in an inert atmosphere, 10-*trans*,12-*cis* gives rise to 11-*cis*,13-*trans* (Fig. 5.1). Upon heating an 11-*cis*,13-*trans* concentrate, 10-*trans*,12-*cis* was produced. Under optimal condi-

tions, an equilibrium is established between these isomers, and only minor quantities of *cis,cis* and *trans,trans* isomers are formed. The isomer shift is actually a thermal [1,5] sigmatropic rearrangement, (Fig. 5.2) allowed according to the orbital symmetry theory (Woodward-Hoffmann). For this sigmatropic rearrangement to occur, it is essential that one of the bonds be in the *cis*-configuration. A similar rearrangement is observed for the isomers 9-*cis*,11-*trans* and 8-*trans*,10-*cis* The phenomenon is actually a tool for chemists to produce new isomers. Any given CLA isomer that contains one double bond in the *cis*-configuration and one in the *trans*-configuration can be heated to be isomerized into another specific *cis,trans* or *trans,cis* isomer. Isomers formed might be predicted from formulae as in Fig. 5.2. A simple rule of thumb is that the two double bonds will move against the *cis* end of the bond pairs. For example, 7-*trans*,9-*cis* (a common isomer in milk fat) will isomerize to 8-*cis*,10-*trans* and *vice versa*. Prolonged heating of isomers

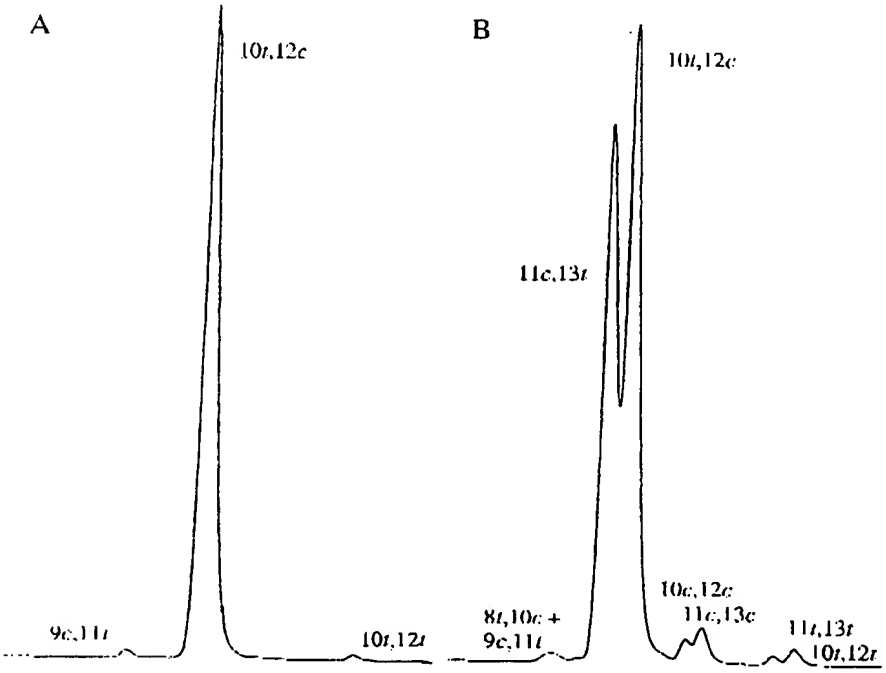

Fig. 5.1. Gas chromatography (GC) profile of ethyl ester of purified 10-*trans*,12-*cis* CLA isomer (a) before and (b) after heating to 220°C in an inert atmosphere for 2 h. The process caused isomerization into the isomer 11-*cis*,13-*trans* by thermal [1,5] sigmatropic hydrogen shift. GC conditions: 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID).

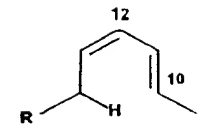

Fig. 5.2. Drawing explain
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Isomer Profile in Avail

The total content of CL
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Stability of CLA Compa

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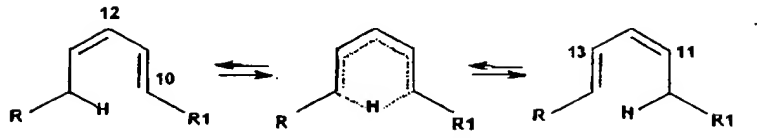

Fig. 5.2. Drawing explaining thermal [1,5] sigmatropic rearrangement between the CLA isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans*. Reaction is spontaneous and the transition state depicted in the middle is not an intermediate product. R= - $(CH_2)_4$ and R_1 - $-(CH_2)_8CO_2H$.

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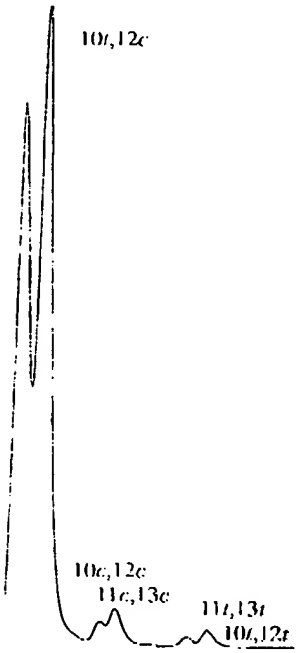


r of purified 10-*trans*,12-*cis* an inert atmosphere for 2 h. 3-*trans* by thermal [1,5] sig- 38 fused silica capillary col-

seems to gradually develop *cis*,*cis* and *trans*,*trans* isomers. Impurities present (iron, copper and other metals) will greatly favor formation of *trans*,*trans* isomers.

Isomer Profile in Available Supplements

The total content of CLA in supplements more or less reflects the starting material. Sunflower oil as a starting material results in ~65% CLA, whereas safflower oil yields up to 80%. Both oils contain a level of palmitic acid that tends to cause precipitation below room temperature. Products are now available with a reduced content of saturated acids and >80% CLA. The products can be classified in two groups, the "4-isomer product" and the "2-isomer product" (Fig. 5.3). The latter product contains almost exclusively 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, both up to ~38% of the oil, or almost 50% of the CLA. The former, however, contains several isomers. The elution order on gas chromatography (GC) of the 4 main peaks is 9-*cis*,11-*trans*; 8-*trans*,10-*cis* (may co-elute with 9-*cis*,11-*trans*);11-*cis*,13-*trans*; and 10-*trans*,12-*cis* (9). In addition a major *trans*,*trans* peak (9,11 and 10,12 co-eluting) often reaches the same level. Such products may contain as little as 8% 10-*trans*,12-*cis*. Despite co-elution, the content of 8-*trans*,10-*cis* can be estimated approximately by measurement of 11-*cis*,13-*trans*. Both are produced to the same degree from their mother components. In other words, the ratio of 11-*cis*,13-*trans* to 11-*cis*,13-*trans* + 10-*trans*,12-*cis* equals that of 8-*trans*,10-*cis* to the co-eluting peak 8-*trans*,10-*cis* + 9-*cis*,11-*trans* (data not published). Products from a single source have been reported to show substantial variation in isomer profile (15), and products also are available that contains virtually no (present data, Table 5.1) or totally lack CLA (10). Two of 17 products sampled and analyzed in January-March 2002 by our laboratory contained high levels of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis* (Table 5.1).

Stability and Breakdown Products of CLA Preparations

Stability of CLA Compared with Linoleic Acid

A few studies report data on the stability of CLA compared with linoleic acid in different test models. Bubbling of oxygen through samples at 90°C resulted in a

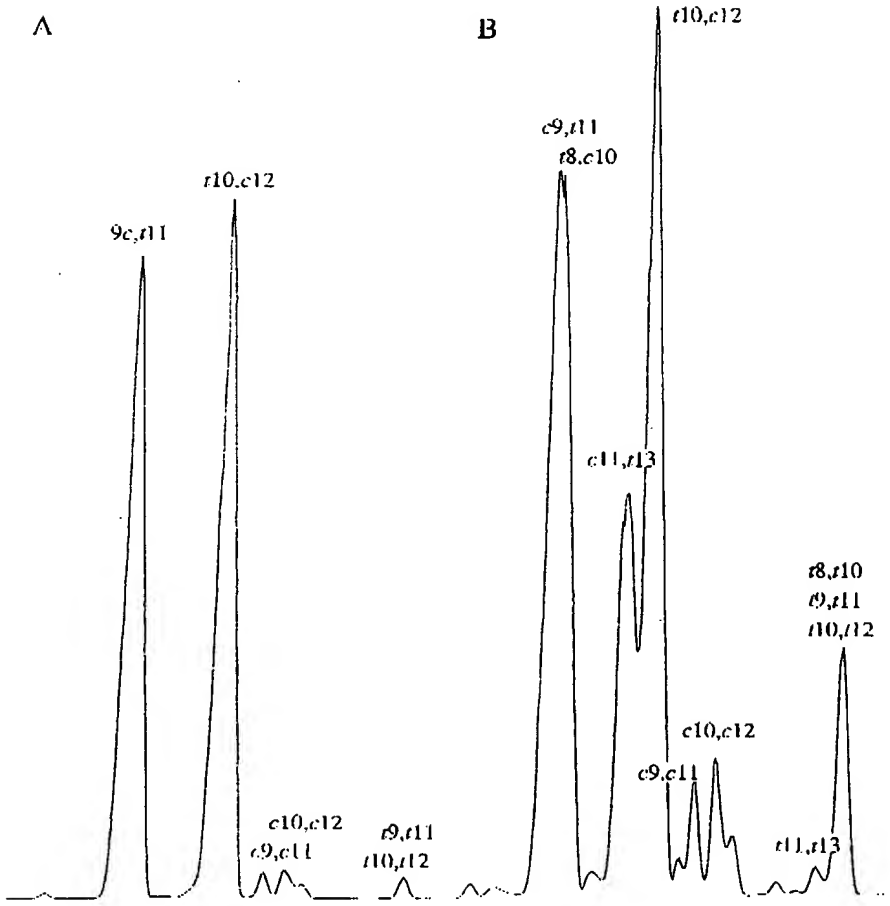


Fig. 5.3. Partial gas chromatography (GC) profile of ethyl esters of (a) a "2 isomer type" and (b) a "4 isomer type" CLA supplement, using a 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID). Product (a) is identical to product No. 14 and product (b) is identical to No.17 in Table 5.1. Note co-elution of 8-*trans*,10-*cis* and 9-*cis*,11-*trans*.

much higher peroxide value (PV) in linoleic acid (16) than for CLA. When a mixture of CLA isomers was heated to 50°C in air, the rate of oxidation was considerably faster for CLA than for linoleic acid. The rate of oxidation was measured as "remaining CLA" by GC. When comparing groups of CLA isomers, stability decreased in order of *trans,trans* > *cis,trans* or *trans,cis* > *cis,cis*. (17). In a study in aqueous and solvent systems measuring stability by the induction period system,

TABLE 5.1
Content of CLA (% of Tot.
January–March 2002[a]

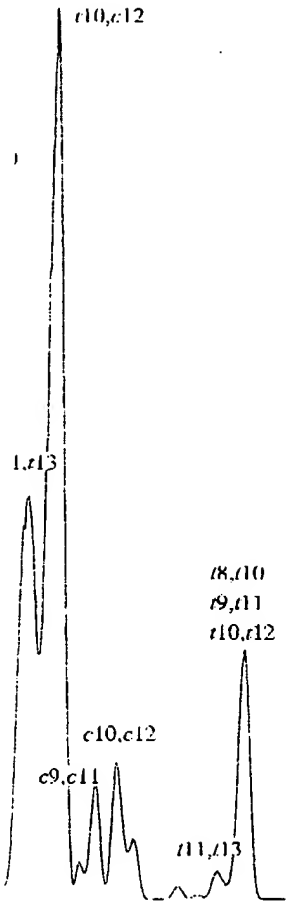
Product	Product type
1	Soft gelatine cap
2	Liquid
3	Soft gelatine cap
4	Soft gelatine cap
5	Soft gelatine cap
6	Soft gelatine cap
7	Soft gelatine cap
8	Soft gelatine cap
9	Soft gelatine cap
10	Soft gelatine cap
11	Soft gelatine cap
12	Soft gelatine cap
13	Soft gelatine cap
14	Soft gelatine cap
15	Liquid, emulsion
16	Soft gelatine cap
17	Soft gelatine cap

[a]The isomers 10-*trans*,12-*cis* and
ucts were of the "4 isomer" type.
9-*cis*,11-*trans* (not tabulated due
trans,12-*cis* in all supplements co
mg KOH/g. (A 100.00% free fatty
region of product 14 and product

CLA was more stable tha
esters (18). Another stud
the following order: oleat
40°C and monitored by
cis,11-*trans*, the major m
and 13-monohydroperox
13-, and 14-monohydrope
Data reported on the
tions. CLA do not easily
seems comparable to that
breakdown of peroxides i

Volatiles

In a pilot project on deve
hexane was observed in
searching for the source



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100-m CP Sil 88 fused silica
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TABLE 5.1
Content of CLA (% of Total) in 17 Commercial Supplements Sampled in January–March 2002[a]

Product	Product type	Country	%CLA	%10*t*,12*c*	%11*c*,13*t*	Acid value
1	Soft gelatine capsule	Norway	80.1	47.8	0.4	197
2	Liquid	Norway	78.6	47.1	1.8	2
3	Soft gelatine capsule	Norway	69.1	46.7	1.2	196
4	Soft gelatine capsule	Norway	78.3	48.7	0.3	197
5	Soft gelatine capsule	Norway	76.4	46.6	1.3	193
6	Soft gelatine capsule	U.S.	71.4	46.3	0.5	189
7	Soft gelatine capsule	U.S.	74.8	43.1	0.9	192
8	Soft gelatine capsule	U.S.	77.9	48.5	0.3	199
9	Soft gelatine capsule	U.S.	70.8	44.4	0.6	189
10	Soft gelatine capsule	U.S.	79.6	45.3	0.4	193
11	Soft gelatine capsule	U.S.	77.0	44.4	2.3	192
12	Soft gelatine capsule	U.S.	74.3	43.6	1.0	187
13	Soft gelatine capsule	U.S.	61.5	28.5	0.8	180
14	Soft gelatine capsule	U.S.	76.3	48.4	0.3	196
15	Liquid, emulsion	U.S.	1.2	47.8	0.3	NA
16	Soft gelatine capsule	S. Africa	51.7	16.5	16.1	198
17	Soft gelatine capsule	Norway	57.7	29.9	16.5	200

[a]The isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans* are expressed as the percentage of total CLA. Only two products were of the "4 isomer" type. Two products were liquids, one oil and one emulsion (1.7% fat). Content of 9-*cis*,11-*trans* (not tabulated due to overlap with 8-*trans*,10-*cis*) is approximately equal or slightly less than 10-*trans*,12-*cis* in all supplements currently available. Distilled products typically have acid values of 195–200 mg KOH/g. (A 100.00% free fatty acid product of oleic acid has a theoretical acid value of 198.60). CLA region of product 14 and product 17 is illustrated in Figure 5.3. NA, not available.

CLA was more stable than linoleic acid as free fatty acids, and less stable as ethyl esters (18). Another study using methyl esters reported that stability decreased in the following order: oleate > CLA > linoleate. Samples were stored in the dark at 40°C and monitored by thin-layer chromatography (TLC), GC and PV. From 9-*cis*,11-*trans*, the major monohydroperoxides formed were identified as 8-, 9-, 12- and 13-monohydroperoxides, whereas 10-*trans*,12-*cis* yielded primarily 9-, 10-, 13-, and 14-monohydroperoxides (19).

Data reported on the PV of CLA preparations are consistent with our observations. CLA do not easily develop high PV, yet the oxidative breakdown of CLA seems comparable to that of linoleic acid. The reason is likely to be a more rapid breakdown of peroxides into secondary oxidation products.

Volatiles

In a pilot project on developing a procedure for CLA production, a high content of hexane was observed in a product by headspace GC-mass spectrometry. After searching for the source of contamination, it was finally concluded that pentane

and hexane are among the secondary oxidation products of CLA. This was later confirmed by experiments. To our knowledge, hexane has never been reported to be an important inherent oxidation product of vegetable oils. In a free fatty acid concentrate of 9-*cis*,11-*trans* stored in the dark with air access for 1 wk, the two major volatiles that developed were, not surprisingly, heptanal and 2-nonenal. The concentration increased from 4.8 and 0.7 to 84.6 and 22.5 μg/g, respectively. Volatile breakdown products seem not to build up in soft gelatine capsule supplements. A CLA product that was stored for 5 y at room temperature contained 2.3 μg/g hexanal and 2.2 μg/g heptanal (data not published). No antioxidant was added to the supplement.

Among less volatile breakdown products, furan fatty acids were reported when air was bubbled through CLA dissolved in a mixture of methanol and water at 50°C. (20). Furanoid fatty acids might also arise in preparation of fatty acid methyl esters (FAME) for GC. To our knowledge, furan fatty acids have not been reported as an oxidative breakdown product in dry oil preparations of CLA.

Polymers

Conjugated oils are considered valuable raw materials for the paint and varnish industry because of their film forming properties ("drying") upon air access. This property gives rise to concern regarding the stability of CLA preparations. In a stability test program, 10 mL of CLA triacylglycerols and free fatty acids were stored in an amber open glass bottle in darkness. After 4 mo at 25°C, controls without antioxidants added were highly viscous and not suitable for further stability testing. The samples had a membrane layer on the surface, and the viscosity clearly developed over time. Samples with antioxidants did show a retarded viscosity development (data not published).

Soft gelatine capsules are considered to give reasonable protection from exposure of unsaturated oils to air. Capsules containing CLA free fatty acids showed a slight increase in polymer content from 1% in freshly prepared capsules to 7% after 5 y (data not published). For comparison of health risks, a limit for rejection on cooking oils has been established in some countries; values listed in a report from the European Parliament are 16% (Holland), and 10% (Belgium and Czech Republic) (21).

Stability of CLA in Soft Gelatine Capsules

No data have yet been published on the stability of CLA in capsules. Observations on polymers and volatiles in capsules are reported above. In a stability test program according to International Conference on Harmonization (ICH) guidelines on a free fatty acid product, the content of total CLA was not significantly reduced after 24 mo at 25°C/60% relative humidity. In this test, CLA was measured by GC. Peroxide value (PV) did not develop in the capsules (data not published).

Next Generation Pr

Isomer Purification

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Triacylglycerols for Foo

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Summary

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Next Generation Products

Isomer Purification

All CLA supplements currently offered contain approximately equal amounts of 9-*cis*,11-*trans* and 10-*trans*,12-*cis*. The extra costs of producing a biased isomer product might be justified if beneficial health effects were documented. The 9-*cis*,11-*trans* and the 10-*trans*,12-*cis* isomers of CLA are now available for research purposes in kilogram scale with a purity of ~90%. In small quantities, purities up to 99% are offered. High yields and high purity can be obtained by repeated crystallization of the methyl ester forms in acetone at temperatures as low as –60°C (22).

A concentrate with 83% 9-*cis*,11-*trans* isomer was obtained from gentle dehydration of ricinoleic acid from castor bean oil and subsequent purification steps (4). The use of urea inclusion compounds does not seem to be a feasible procedure to separate 9-*cis*,11-*trans* and 10-*trans*,12-*cis* (23). Enzymes, however, are promising tools for these separations. A 98% concentrate of 9-*cis*,11-*trans* was reported by using lipase from *Geotrichum candidum*. The enzyme was capable of esterifying selectively 9-*cis*,11-*trans* to monohydric alcohols from a mixture of several isomers (24). A patent has been issued on purification and characterization of isomerases from *Propionibacterium acnes* and *Clostridium sporogenes*. The purified isomerase preparations were able to quantitatively isomerize linoleic acid into the 10-*trans*,12-*cis* isomer of CLA (25).

Triacylglycerols for Food Applications

Free fatty acids and monoalkyl esters are applicable to supplement capsules and probably also to animal feed formulations. However, as an ingredient in food for human consumption, CLA is most attractive as a triacylglycerol. A nonspecific lipase has been reported to esterify CLA with glycerol very efficiently (26). Incorporation of CLA into food fats and oils has also been reported for fish oils (27), butterfat (28,29), and corn oil (30). A bottled triacylglycerol product, stabilized with antioxidants, has been available in the health food market in Scandinavia since 2000. Flavor and antioxidants are added to the oil designed to be taken by spoon. Further technical developments of CLA products improving the stability and applicability as well as addressing specific issues of food legislation will require attention before CLA can be made available as an ingredient for animal feed and human food.

Summary

CLA supplements for human consumption have been available since 1995, and most of the products contain between 60 and 80% CLA in the form of free fatty acids. The history of CLA produced for technical purposes dates back almost 100

y, however. The isomer profile of the supplements range from an almost pure 9-*cis*,11-*trans* + 10-*trans*,12-*cis*-50/50 mixture (made in alcohol solvents between 100 and 150°C), to a mixture with four prominent *cis,trans* or *trans,cis* isomers produced in high alkaline water at high temperatures, of which 8-*trans*,10-*cis* and 11-*cis*,13-*trans*-18:2 are produced from 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, respectively, by thermal [1,5] sigmatropic rearrangements of the isomers. Supplements are typically offered as free fatty acids in soft gelatine capsules. Unpublished data on stability of CLA in capsules stored according to ICH guidelines for 2 y did not show any loss of active ingredient.

Acknowledgments

Per Christian Szebo and his staff at the laboratory of Natural ASA is acknowledged for patient experimental work on CLA production and purification process developments for the last 5 years. Thanks to Prof. emeritus Lars Skattebøl for valuable comments on migration of sigma bonds.

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